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Legionnaires Disease: Historical Perspective

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INTRODUCTION

The publicity has subsided. As we have learned about the illness that struck members of the Pennsylvania American Legion in the summer of 1976, most of the mystery is gone. We were spared the "monster killer" that was constructed by the press in 1976, only to have it appear a few years later in the form of human immunodeficiency virus and acquired immunodeficiency syndrome. Legionella and human immunodeficiency virus have taught us the value of the vast medical and scientific resources that we have developed, but we have also learned humility in the face of resourceful and seemingly indefatigable microbial foes.

If we do not yet have all of the answers about *Legionella* species, a great deal of information has been accumulated about virtually every aspect of the bacteria and the infections they cause. On the 10th anniversary of the publication of the description of the Philadelphia epidemic (101, 175), it is appropriate to consider past progress, assess current status, and reflect on future directions. I will not attempt a comprehensive review of the now voluminous literature, but rather will select pertinent references that illustrate the contrast between our present knowledge and the rudimentary information that was available 10 years ago.

We have experienced two large epidemics of *Legionella pneumophila* infection in Burlington, Vt. The first occurred in 1977 at a time when the outlines of the microbiological and clinical aspects of the infection were known, although not formally published, but epidemiological and environmental data were minimal. The nature of the infectious process was suspected by astute clinicians (11). Most of the infections were diagnosed by immunological techniques at the Centers for Disease Control (CDC). The bacteria were a mystery to clinical laboratories, but investigators at CDC had already discovered that they could be cultivated on Mueller-Hinton agar supplemented with IsoVitaleX (BBL Microbiology Systems) (93). A single isolate was made by inoculation of post-mortem lung tissue onto agar that investigators from CDC brought for the investigation.

In contrast, the second epidemic in 1980 occurred after we had had substantial experience with *Legionella* spp. (157). Buffered charcoal-yeast extract agar and selective media (202) had just been introduced into the laboratory through the kind help of James Feeley. Direct immunofluorescence conjugates were available from CDC, and the possibility of detecting *Legionella* antigen in urine had been demonstrated. Although the summer of 1980 was a hectic one, we were dealing with a microbiological problem for which we were prepared, rather than an unknown and elusive agent. The difference from our experience 3 years earlier was dramatic. In 1987, communities that are faced with epidemics have even greater microbiological resources, including the ability to assist epidemiologists by molecular analysis of strains.

Philadelphia Epidemic of Legionnaires Disease

One must consider events in a temporal context to understand the furor that the first recognized epidemic of *Legionella* infection created. In the summer of 1976, attention was focused on the bicentennial celebration of the Declaration of

Independence and Philadelphia was at the center of the action. There could not have been a better place to stage a medical media event. The previous winter an isolate of influenza virus A with the antigenic characteristics of the original strain recovered from pigs had been identified at Fort Dix, N.J. Although no virus was isolated during the devastating influenza pandemic of 1918, serological surveys suggested an isolate similar to the swine strain. The reappearance of such a virus raised fears of another worldwide epidemic. Influenza, which is usually a winter disease, had made an early appearance at the onset of the 1918 epidemic, and doomsayers warned of a similar pattern in 1976. Thus, newspaper reports of a mysterious, fatal disease that resembled influenza clinically and was of epidemic proportions aroused enormous concern.

All together, 182 members of the Pennsylvania American Legion developed an acute respiratory illness, and 29 individuals died after they returned from the convention in Philadelphia. The epidemiological and microbiological studies were to continue for 6 months before the cause of the disease was announced. Much of the basic framework of our knowledge of Legionnaires disease, as the epidemic came to be known, was developed during the intensive investigations that were conducted by a team from CDC and the Pennsylvania Department of Health (101). Only the source of the disease remained a mystery, because laboratory support for environmental investigations did not exist. The sharply peaked epidemic curve did suggest, however, that a common-source epidemic had occurred.

There are many lessons to be learned from the Philadelphia outbreak. First and foremost was the dismissal of the idea that infectious diseases are a thing of the past or even that all infectious diseases are known. In fact, the decades of the 1970s and 1980s have seen a host of "new" infectious diseases: some caused by newly recognized organisms, others caused by traditional pathogens in new clothes. One can point to staphylococcal toxic shock syndrome, Lyme disease, the African hemorrhagic fevers, *Campylobacter* enteritis and gastritis, and of course acquired immunodeficiency syndrome.

It is quite clear that the magnitude of the Philadelphia epidemic contributed to the recognition of *Legionella* species as important human pathogens. As we soon learned, members of the genus had been isolated some 25 years earlier from sporadic cases. Whether the intense public scrutiny from the press and the Congress contributed to the solution cannot be known with certainty. Ironically, Joseph McDade, the investigator who cracked the riddle, had just returned from several years in the Middle East, where he had shed the habit of reading newspapers and listening to television news (150).

A second lesson is the difficulty of breaking out of traditional patterns of thought when we are confronted with the unknown. Initially, viral and toxic etiologies were sought, because of the clinical resemblance of the pneumonia to severe influenza. Although clinical features suggestive of *Legionella* infection, such as prominence of gastrointestinal and cerebral symptoms, have been described (121, 156, 182, 231), some investigators have found it difficult to differentiate between pneumonia caused by *Legionella* spp. and that caused by other bacteria (186, 222, 279). The differential diagnosis must be much broader when the clinical presentation is not pathognomonic. As recently as 1985, influenza was the initial diagnostic consideration during an outbreak of Legionnaires disease in England, because of the clinical presentation and the presence of antibodies to influenza virus in the first sera collected (211). Even after the etiologic agent had been announced by CDC in 1977, discussion about toxic causes continued (41, 239).

Pathological analysis is subject to the same difficulties. A panel of experts in pulmonary pathology was unable to suggest a bacterial etiology, because of the preponderance of macrophages in the airspaces of some patients, because of the likelihood that the purulent inflammation observed was caused by superinfecting bacteria, and because bacteria were not demonstrated with the histological stains that are traditionally applied to tissue sections (36). When bacteria that had the morphology of gram-negative bacilli were subsequently demonstrated by electron microscopy in lung tissue from fatal cases, the significance of the observation was obscured by the consideration that they were secondary invaders (F. Murphy, personal communication). The difficulty of stretching beyond the bounds of the known will always be with us. Muder and colleagues have pointed out that investigators concentrated their attention on cooling towers as an environmental source of *Legionella* epidemics until showers were demonstrated to be a possible source, after which potable water received most attention (185). In future investigations of epidemics, it is important to recognize that the apparent menu of sources for the infection may not even include the true site.

The process by which the etiologic threads from the Philadelphia epidemic were unraveled is also instructive. Serendipity undoubtedly played a role in the discovery; in all likelihood, the bacterium would not have been identified if specimens had not been tested in a laboratory that routinely used guinea pigs for the recovery of rickettsiae. It is important to recognize, however, that without the comprehensive reference laboratories maintained at CDC, serendipity would not have been possible. Targeted research works well if one knows the answer at the outset.

Many investigators contributed to the Philadelphia studies, all of whom were important to the final outcome. But in the end it was the conscientiousness and persistence of one man, Joseph McDade, that led to the discovery of the Legionnaires disease bacterium, subsequently named *Legionella pneumophila*. Two months of work in August and September of 1976 had provided some tantalizing clues. Guinea pigs inoculated with lung tissue became ill and a few gram-negative bacilli were seen in a Gram stain of tissue, but the disease could not be transmitted to other guinea pigs effectively and the organism could not be subcultured onto agar. Attempts to isolate the bacterium in embryonated eggs failed, probably because the procedure for isolation of rickettsiae includes penicillin and streptomycin to suppress "contaminants." The critical event was McDade's return to the problem during the week between Christmas and New Year's Day (150). He had been bothered by the unexplained observations and resolved to identify the gram-negative bacilli he had seen. This time he omitted the antibiotics from the inoculum when he infected eggs, because he thought he might well be interested in the contaminant. The rest is history. Bacteria were identified by the Gimenez stain, which was developed for rickettsiae but contains carbol fuchsin and stains *Legionella* spp. well. Application of the indirect immunofluorescence technique to the sera of patients from Philadelphia indicated that an antibody response had been mounted against the newly isolated bacterium. McDade wanted more time to be sure, but the pressure for an answer was too great. Fortunately, the CDC investigators had done their work well and they were right. A whole new family of pathogenic bacteria had been found.

HISTORY AND PREHISTORY

With a bacterium in hand, diagnostic tools could be developed. It became apparent rather quickly that neither the bacteria nor epidemics were new. In the archives of CDC unsolved epidemics may be put aside, but it is hard to forget them. Outbreaks of acute respiratory disease from as far back as 1959 have now been attributed to *L. pneumophila* (197, 244, 248). The two most instructive events had occurred in the 1960s. An epidemic of respiratory disease at St. Elizabeth's hospital in Washington, D.C., in 1965 raised the question of airborne dissemination of bacteria; epidemiological investigation had suggested an association with open windows and nearby construction (248).

The most memorable of the unsolved episodes was the acute respiratory disease that afflicted employees and visitors in a health department in Pontiac, Mich. (114). The disease, which had been dubbed "Pontiac fever," differed greatly from the Philadelphia epidemic of Legionnaires disease. Although respiratory symptoms were prominent in both outbreaks, pneumonia was not observed in Pontiac. There were no fatalities, whereas 29 of 182 stricken Legionnaires died. The mildness of the illness in Pontiac might suggest a low bacterial inoculum, yet the attack rate among health department employees was 95%. Epidemics of Legionnaires disease have an attack rate of <5%. Furthermore, the incubation period of illness in Philadelphia was 2 to 10 days, as opposed to a mean of 36 h in Pontiac. A second, less well-documented epidemic of nonpneumonic illness was identified by serological techniques in workers at a steam turbine plant (100). Thus, very quickly investigators recognized that two distinct clinical and epidemiologic syndromes could be caused by *L. pneumophila*. The Pontiac outbreak still provides the best documentation that *Legionella* spp. can produce epidemic illness after dissemination in aerosols.

Although there were no fatalities in Pontiac, the CDC investigators who entered the building to study the disease were numbered among the victims. When you get sick yourself, you do not forget easily, and work continued for 3 years. In retrospect, it is amazing how close to the answer these investigators got; the reason for the failure to close the circle is inexplicable (152). The very high attack rate and the tight epidemic curve in Pontiac suggested an airborne route of spread. As a part of the investigation, sentinel guinea pigs were exposed to the air-conditioning system and animals were inoculated with water from the condenser. The animals developed a nodular, focal pneumonia, in which gram-variable bacilli were demonstrated. Administration of antibiotics partially protected the guinea pigs against illness. Once again, serendipity played a part; if mice, rats, or rabbits had been used, clinical illness would not have been observed in the animals and the agent might not have been isolated. Back in Atlanta, guinea pigs developed acute pneumonia after they were exposed to an aerosol of water from the condenser of the air conditioner in Pontiac. When the water was autoclaved or filter sterilized, no lesions could be produced after generation of an aerosol. Chicken embryos died after inoculation of the eggs with tissue from the guinea pigs, and gram-negative bacilli were seen in impression smears from the infected eggs. Only the final test, the key to the successful studies in 1976, failed. When the sera from patients in Pontiac were tested by indirect immunofluorescence for antibodies to the egg-grown bacterium, a consistent pattern of reactivity was not apparent. The reason for the inability to detect those antibodies, which were present when retested years later, is unknown, but it prevented the investigators from concluding that they had isolated an etiologic agent.

The Philadelphia experience has also assisted subsequent investigators in identifying other etiologic agents of pneumonia within the genus. The most important of these agents is *Legionella micdadei*. In 1979, investigators in Charlottesville, Va. (219), and Pittsburgh, Pa. (187), recognized the presence of an acid-fast bacterium that could not be cultivated on traditional media for mycobacteria and nocardia. Furthermore, the pneumonia that was produced in immunosuppressed patients was characterized by an acute rather than a granulomatous inflammatory response. This important pathogen would not have been recognized if a battery of "special stains" had not been applied to tissue obtained from severely immunocompromised patients, such as individuals who had received renal transplants, regardless of the histological response. Pathologists and microbiologists have an obligation to discourage indiscriminate use of increasingly precious resources, but it is also important that we not narrow our vision too severely when the stakes are high, the susceptibility to infection is great, and the predictability of the organism or inflammatory reaction is low. The Pittsburgh group was able to isolate an organism, using techniques that the CDC had developed for *L. pneumophila*.

The bacterium that had been isolated in Pittsburgh (202) was initially called the "Pittsburgh pneumonia agent," but it was soon demonstrated that it was a new species of *Legionella* and that it had been isolated previously (124, 202). In fact, the earliest recorded isolate of a *Legionella* species was a strain of *L. micdadei* that was recovered by Hugh Tatlock in 1943 during an outbreak of Fort Bragg fever. This epidemic infection was accompanied by a distinctive pretibial rash that resembled erythema nodosum. Although a pretibial rash has been described in a case report of *L. pneumophila* infection (126), the rash did not resemble erythema nodosum. Skin lesions have not been a part of the clinical spectrum of Legionnaires disease (156). Tatlock has stated the case from a first-hand viewpoint that Fort Bragg fever was indeed caused by *Leptospira autumnalis* and that the *Legionella* isolate recovered from the blood of a sick soldier was an adventitial agent (242). The source of the isolate is unknown. Although antibodies that react with legionellae can be detected in a variety of animal species (48), isolation of the organism has been rare (46). We have never detected antibodies to *L. pneumophila* in uninfected guinea pigs during 8 years of work with a guinea pig model of the infection (G. S. Davis and W. C. Winn, Jr., unpublished observations). It is possible, however, as suggested by Tatlock, that the organism was introduced from an environmental source in the time between collection of the specimen and inoculation of the guinea pig. Isolates of species other than *L. micdadei* had also been made before 1976, and Joseph McDade was not the only rickettsiologist to stumble on the genus. During her studies of rickettsial disease at the National Institutes of Health, F. Marilyn Bozeman isolated two organisms that subsequently turned out to be the first recorded isolate of *L. pneumophila* (the OLDA strain in 1949) (173) and *Legionella bozemannii* (the WIGA strain) (124). In 1959, she also isolated a bacterium (HEBA) that is a strain of *L. micdadei* (124).

TAXONOMY AND NOMENCLATURE

Life was simple 10 years ago. At the time of the first international symposium on *Legionella* held in 1978 at the CDC, there was a single genus and species, referred to as the

Legionnaires disease bacterium until a proposed nomenclature was presented at the symposium. Brenner and associates used a variety of techniques, most prominently deoxyribonucleic acid (DNA) homology, to establish that the newly recognized pathogen indeed represented a new family (*Legionellaceae*), genus (*Legionella*, after the Philadelphia victims), and species (*pneumophila*, after the predilection of the bacterium for the lung) (30). Serological diversity within the species had already been recognized by the time the nomenclature was established (178). In the succeeding 10 years, a plethora of serotypes and species has been described (Table 1) (27, 29). If the complexity of the genus does not yet approach that of *Salmonella*, it is already considerably more diverse than most established genera.

Bacterial taxonomy is based on the evaluation of multiple sets of data, including phenotypic, immunologic, and genotypic characteristics. Traditional phenotypic analysis of *Legionella* by determination of biochemical reactivity is not very useful, either for diagnosis or for classification. Serological reactions, determination of cellular fatty acids, and isoprenoid quinone analysis are useful for classification, but do not differentiate accurately all species and are not routinely available for diagnosis in the clinical laboratory (27). Determination of DNA relatedness has been the mainstay of classification. As Brenner has pointed out, however, phenotypic characteristics ensure that the genetic classification at the species level is relevant to everyday life; in particular, the gram reactivity and morphology of the bacteria, growth on buffered charcoal-yeast extract agar in the presence of cysteine and alpha-ketoglutarate (BCYE-a), and absence of growth on blood agar or BCYE-a agar without cysteine should serve to alert the microbiologist that a member of the genus *Legionella* may have been isolated (27).

TABLE 1. Classification of *Legionella* species

Species	Serogroup	Isolated from:		Reference
		Humans	Environment	
L. pneumophila	1	Yes	Yes	101
	2	Yes	Yes	178
	3	Yes	Yes	178
	4	Yes	Yes	178
	5	Yes	Yes	86
	6	Yes	Yes	179
	7	Yes	Yes	19
	8	Yes	No	21
	9	Yes	Yes	70
	10	Yes	Yes	180
	11	Yes	No	249
	12	Yes	Yes	251
L. bozemanii	1	Yes	Yes	28
	2	Yes	No	241
L. micdadei		Yes	Yes	125
L. dumoffii		Yes	Yes	28
L. gormanii		No	Yes	183
L. longbeachae	1	Yes	No	177
	2	Yes	No	20
L. jordanis		Yes	Yes	42
L. oakridgensis		No	Yes	196
L. wadsworthii		Yes	No	71
L. feeleii	1	Yes	Yes	128
	2	Yes	No	252
L. sainthelensi		No	Yes	35
L. anisa		No	Yes	116
L. maceachernii		Yes	Yes	29
L. jamestowniensis		No	Yes	29
L. rubrilucens		No	Yes	29
L. erythra		No	Yes	29
L. hackeliae	1	Yes	No	29
	2	Yes	No	271
L. spiritensis		No	Yes	29
L. parisiensis		No	Yes	29
L. cherrii		No	Yes	29
L. steigerwaltii		No	Yes	29
L. santicrucis		No	Yes	29
L. israelensis		No	Yes	13

TABLE 2. Clinical features of Legionnaires disease and Pontiac fever[a]

Sympton	% of patients	
	Legionnaires disease	Pontiac fever
Cough	75	46
New sputum production	45	
Dyspnea	50	
Hemoptysis	21	
Myalgias	38	95
Upper respiratory symptoms	13	
Headache	32	88
Confusion	45	19
Nausea or vomiting	30	10
Diarrhea	33	21
Abdominal pain	8	
Fever		86
Fever above 39°C	70	
Relative bradycardia	40	

[a] Adapted from reference 7.

The short life of *Legionella* taxonomy has not been totally without controversy. There is no disagreement on the need for a new family of bacteria or on the legitimacy of the species designations as published. Nor is there disagreement about the phenotypic and genotypic characteristics on which the division of genera into species is made. There is argument, however, about the degree of genetic divergence between two isolates that justifies the creation of a separate species. Garrity and associates have suggested that the Pittsburgh pneumonia agent be given species designation as *Tatlockia micdadei* and that the species with blue-white autofluorescence be placed in the genus *Fluoribacter* (109). These investigators have not addressed the classification of other species that have been identified subsequently. Brenner has commented that such disputes must be adjudicated historically by the frequency with which the terms are used by the scientific community (27). At present the majority of authors have accepted the validity of a single genus, *Legionella*, within the family *Legionellaceae*, in part perhaps because the CDC classification is the only one that embraces all of the characterized species.

LEGIONELLA INFECTIONS

Clinical Studies

Two distinct clinical syndromes have been associated with infection by *Legionella* species. The presentation of the nonpneumonic illness Pontiac fever was clearly defined by the initial outbreak (Table 2) (114).

The broad outlines of the clinical presentation of the pneumonic illness Legionnaires disease were sketched in Philadelphia, and the picture was virtually completed in the epidemics that soon followed (11, 101, 156) (Table 2). In most cases the onset is acute, but it may be difficult to identify the onset in hospitalized patients who have complex

medical problems. The infection is accompanied by a high fever that increases in a stepwise fashion; shaking chills tend to recur. Extrapulmonary symptoms are common. Such complaints as fatigue, malaise, myalgia, and arthralgia may suggest the diagnosis of viral infection and mislead the clinician (211). Gastrointestinal complaints, including nausea and diarrhea, and symptoms referable to the central nervous system, including headache and confusion out of proportion to the degree of fever, occur in a minority of patients, but may suggest the diagnosis. Many of the symptoms, including cough, are common to Legionnaires disease and Pontiac fever. Sputum is not always produced and tends to be nonpurulent. Similarly, legionellae are not usually demonstrable in respiratory secretions. But on occasion secretions may be purulent and bacteria may be demonstrated, especially if material is obtained directly from the lung (163, 273). Although it has not been reported in all series, minor degrees of hemoptysis may be present.

Radiographic Appearance

The radiographic appearance is variable. Most commonly, patchy unilateral infiltrates are seen initially. Extension of the infiltrates produces consolidation in one or more lobes; infiltrates may be bilateral in as many as two-thirds of patients (91, 155). In some cases distinctive nodular lesions, which resemble those produced in some fungal infections, are produced (Fig. 1) (56). Small pleural effusions are common; large effusions and empyema occur but are distinctly unusual. Abscesses are not often demonstrated by radiographic techniques, although they can be found rather frequently in pathologic specimens from fatal cases (274). The resolution of radiographic infiltrates often lags behind clinical recovery. Complete resolution requires many months, and convalescence may also be protracted, even in previously healthy individuals.

Pathology and Complications

When the lungs are examined macroscopically, a multifocal pneumonia with a tendency for coalescence of lesions is most common (274). The exudates contain large amounts of fibrin, producing a granular white appearance after fixation with formaldehyde solution; inflammatory material is easily scraped from the cut surface of the lung for examination in the light or fluorescent microscope. In a small percentage of cases, the lesions are lobar and difficult to distinguish from classical lobar pneumococcal pneumonia. The nodular macroscopic lesions are distinctive pathologically, as well as radiologically.

Microscopically, there is an abundant inflammatory exudate in the distal airspaces (alveoli, respiratory bronchioles, and terminal bronchioles) of most cases. Proximal bronchioles and bronchi are spared. The reason for the discrep-

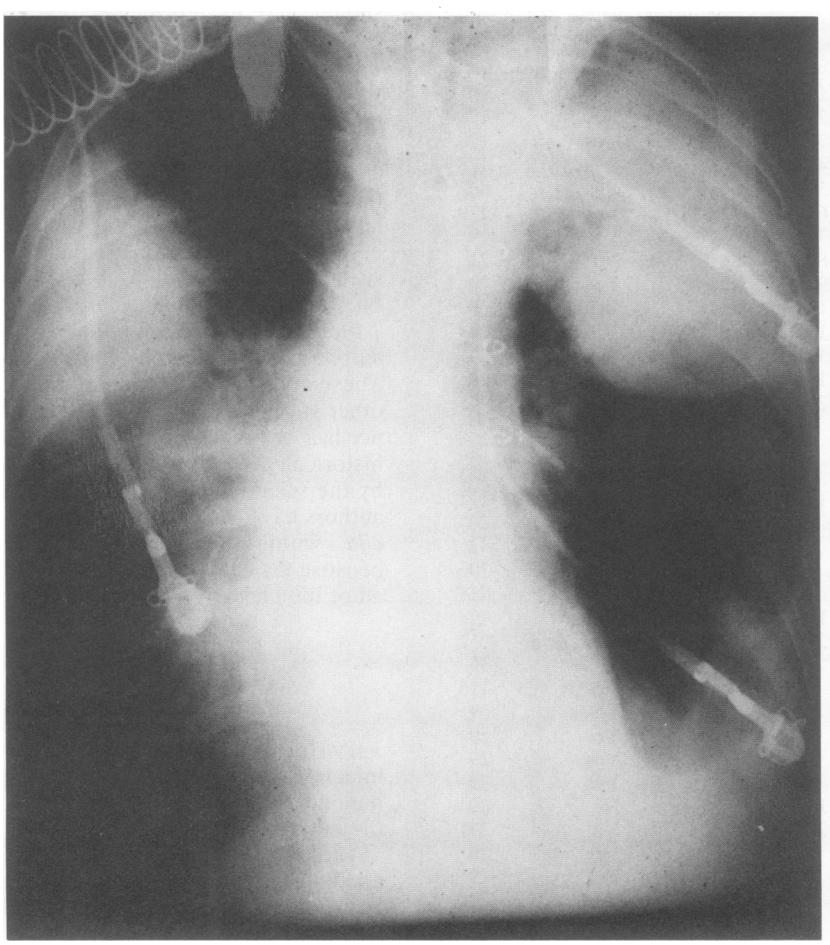

FIG. 1. Chest X ray of a patient with acute *L. pneumophila* pneumonia. Well-demarcated peripheral densities are present. These distinctive lesions are present in a minority of patients, but suggest the diagnosis when present.

ancy between the amount of inflammation in the lungs and that in expectorated sputum is unclear. The exudate is composed of a mixture of polymorphonuclear neutrophils (PMNL) and macrophages, with one or the other predominating in some cases. A characteristic, but not pathognomonic, lysis of the exudate is often seen and may suggest the correct diagnosis. In a small minority of lungs, the airspaces are relatively acellular and are filled with proteinaceous material. Diffuse alveolar damage with hyaline membranes is not uncommon and has been described as the sole abnormality in a lung biopsy (194), but other potential causes of alveolar damage are often present.

The lesions in pneumonias caused by species other than *L. pneumophila* appear similar. Data are most extensive for *L. micdadei* (274), but the brief descriptions of radiographic and pathologic abnormalities in pneumonias caused by other species suggest that they are very similar. Nodular lesions have been described in lungs infected by *L. micdadei* (210). The extent of radiographic disease has been correlated with the demonstration of legionellae by direct immunofluorescence of sputum (159), a finding that also correlates with increased numbers of colonies recovered by culture.

After the description of the initial outbreaks, subsequent reports have primarily contributed information about unusual complications. We now know that dual infections with multiple species (60), multiple serotypes, or even multiple subtypes (148) may occur. Pleural effusions may rarely occur in the absence of obvious pneumonia (181). Subclinical infection has been documented serologically (63) and may be more common than is realized. Furthermore, diverse clinical expressions of disease may occur after exposure to the same environmental source of bacteria (112). It is tempting to refer to nonpneumonic disease as Pontiac fever, but this designation is probably best reserved for outbreaks that conform to both the epidemiological and the clinical definitions.

Legionella bacteremia has been demonstrated (74) and may be relatively common in severely ill patients (90, 216). Symptoms referable to organ systems other than the lung are common, but extrapulmonary inflammatory lesions are uncommon. A variety of well-documented cases of extrapulmonary disease have been described, however, including pyelonephritis (58), hepatic abscesses (51), cerebral microglial reactions that resemble granulomata (51), pericarditis (171), endocarditis (172), and hemodialysis fistula infections (149). In most cases, *L. pneumophila* was the only bacterial pathogen isolated, but *L. pneumophila* isolated from a perirectal abscess along with a mixture of anaerobic bacteria has been described (2). Pneumonia has usually been recognized before or concurrent with the extrapulmonary lesions, but, as with pleural effusions, a few cases have been described in which disease of the pulmonary parenchyma was not obvious (190).

Extrapulmonary inflammatory lesions have been described less frequently in infections caused by legionellae other than *L. pneumophila*. A cutaneous abscess has been produced by *L. micdadei* (1).

It is not surprising that permanent pathologic sequelae should result from a necrotizing pneumonia in which microabscess formation is common. The most convincing description of pulmonary fibrosis as a complication of *L. pneumophila* infection has been provided by Chastre and associates, who described five cases from a nosocomial epidemic, in which interstitial or intra-alveolar fibrosis or both resulted (39). The frequency with which *Legionella* sp. contributes to pulmonary fibrosis is unclear, but such permanent damage may contribute to persistent deficits in pulmonary function (39, 162).

Legionellae are more difficult to demonstrate with chemical stains in tissue than in smears. In most laboratories the tissue versions of the Gram stain do not stain the organism well, even when a Gram stain of tissue imprints colors many bacteria. The organisms are not usually visible with hematoxylin-eosin and are not stained by the periodic acid-Schiff reaction or the Grocott modification of the methenamine silver stain for fungi. The Warthin-Starry and Dieterle stains for spirochetes are sensitive stains for all bacteria and may demonstrate the legionellae when the Gram stain is negative (37, 257). *L. micdadei* is acid fast in tissue (187) and has been demonstrated in pulmonary secretions by the modified Kinyoun stain (222); for unknown reasons, the bacteria are no longer acid fast after they have been grown on agar media. Reports of unusual clinical or pathologic manifestations of *Legionella* infections should be viewed with a critical eye focused on the means by which the etiologic agent was documented. In 1976, the difficulty of culturing the organism forced us to rely on immunologic techniques. In 1986, recovery of legionellae in culture is routine and at this date remains unequivocal proof of etiology, because a carrier state for these organisms in humans has not been demonstrated by culture (31). Direct immunofluorescence of bacteria in secretions and tissue and detection of serum antibodies are useful diagnostic tests. Cross-reactions with other bacteria do exist, however, and documentation by these tests should not be considered definitive. During an epidemic situation demonstrations of bacteria in tissue by immunofluorescence or a seroconversion (fourfold or greater increase in titer of antibody to at least 1:128) are credible. Seroconversion in cases of sporadic disease is an ever so slightly tarnished diagnostic modality and demonstration of a single, high antibody titer is inadequate.

Antimicrobial Therapy

Analysis of the clinical records of the Pennsylvania Legionnaires suggested that erythromycin was the most effective therapeutic agent used (101), and statistical confirmation of this result was achieved during the first Vermont epidemic (32). It is ironic that an unfashionable antibiotic, such as erythromycin, won the prize; one can only speculate how long it would have taken to garner the information if all of the Legionnaires had been hospitalized in tertiary medical centers stocked with a panoply of new antibiotics.

The clinical response to erythromycin therapy may occur immediately or be delayed for several days. Relapses of *L. pneumophila* infection are well documented (121), especially after oral medication or intravenous therapy in low doses. Clinical failure has also been described in pneumonia caused by *L. bozemanii* (199) and *L. micdadei* (222). Strains of *Legionella* species that are naturally resistant to erythromycin in vitro have not been reported, although resistant mutants have been elicited in the laboratory (62). Erythromycin is bactericidal in vitro (64), but several investigators have demonstrated that it is only bacteriostatic in cultured monocytes (140, 260). For these reasons and because of the severe phlebitis that accompanies intravenous administration of erythromycin, the search for alternative therapeutic agents has continued.

The susceptibility of *Legionella* species to most antibiotics can be tested readily in vitro by agar dilution or broth dilution methods. Unfortunately, the results correlate very poorly with clinical efficacy, and there is little sense in pursuing this route except as a preliminary screening procedure for new antibiotics. Even bactericidal testing in vitro

does not discriminate between clinically effective and ineffective antibiotics (64).

Fraser and colleagues at CDC reported the first attempt to use an animal model for susceptibility studies (102). These investigators treated guinea pigs that had been inoculated intraperitoneally with *L. pneumophila* and found that erythromycin and rifampin prevented fatal infection. Clinically ineffective antibiotics, such as penicillin and gentamicin, were also ineffective in the guinea pig model. These results were confirmed and extended by Pasculle (201) and Edelstein (72), who inoculated guinea pigs with *L. pneumophila* or *L. micdadei* by the intratracheal route, which more closely simulates human infection than does intraperitoneal infection (151). Rifampin appears to be more effective than erythromycin in experimental models, but cannot be used as the sole therapeutic agent in humans because of the high frequency with which resistance is induced (61). In the animal models trimethoprim-sulfamethoxazole was also effective, and there are anecdotal reports of its successful use in humans (156).

Cure by a variety of antimicrobial agents has been reported, but the results are difficult to evaluate in uncontrolled studies with small numbers of patients. The most promising new agents are the quinolone derivatives, which have demonstrated effectiveness equal to or greater than that of erythromycin in tissue culture and animal systems (224, 225). It may be difficult to justify and even more difficult to mount controlled clinical trials of new agents against legionellae. Rifampin has been used as a second therapeutic agent in patients with severe disease or poor response to erythromycin therapy. Promising new agents may play a similar adjunctive role and may become invaluable in the unfortunate event that bacterial resistance to erythromycin develops.

Assay of antimicrobial agents in cultured macrophages may be a less costly substitute for whole animal models (140, 260), but more experience with this system is needed. It will be difficult to assess the capability of antibiotics that are rapidly bactericidal to extracellular bacteria.

Preventive therapy with oral erythromycin has been reported to be effective in preventing *Legionella* infection in renal transplant patients (258), but should be reserved for such highly susceptible patients during an epidemic that has not yet been controlled by elimination of environmental bacteria.

The mechanism for the dichotomy between in vitro susceptibility and clinical response to antimicrobial agents is unknown. Beta-lactamases are produced by a number of *Legionella* species (29), despite their in vitro susceptibility to beta-lactam antibiotics, but these agents are also not effective in vivo against *L. micdadei*, which does not produce a beta-lactamase. Perhaps the most attractive suggestion is that ineffective antibiotics do not penetrate well into macrophages, a prime site for growth of legionellae in tissue culture (138) and animal tissues (52).

MICROBIOLOGY AND IMMUNOLOGY

Members of the *Legionellaceae* are gram-negative, aerobic, nonsporeforming, non-acid-fast (with the exception of *L. micdadei* in vivo), unencapsulated bacilli that measure 0.3 to 0.9 μm in width and 2 to ≥20 μm in length (27). Bacilli in tissue are only rarely filamentous, but elongated forms may predominate in some culture media (205). They are nonsaccharolytic, but most species produce a protease which liquefies gelatin. Branched-chain fatty acids predominate in the cell wall, and the bacteria contain large amounts of ubiquinones with more than 10 isoprene units in the side chain (27). All species contain either catalase or peroxidase (29, 207), but the reaction may be weak compared with that produced by other catalase-containing bacteria. The guanine-plus-cytosine content of the DNA is 38 to 52 mol%. DNA relatedness among the various species ranges from 0 to 67%.

The major source of energy for growth of the family appears to be from amino acids (110, 153, 246). Some amino acids are essential for growth. The precise identification of the essential compounds has varied among reports, perhaps reflecting differences in the composition of the medium. Amino acids are catabolized via the Krebs cycle, and sugars are synthesized by the gluconeogenic enzymes of the Embden-Myerhof-Parnas pathway (110). An energized mechanism was demonstrated for the uptake of glutamate by the bacterial cells, but an energized mechanism for glucose uptake could not be demonstrated. The presence of metal ions, notably ferric iron, enhances growth, but may not be absolutely essential (212, 245). Siderophores are compounds that enhance the solubilization and transport of iron, when that metal is in short supply. Reeves and colleagues were unable to identify the commonly recognized phenolic or hydroxamate siderophores (213).

The first agar medium demonstrated to support the growth of *L. pneumophila* was Mueller-Hinton agar supplemented with IsoVitaleX (93). A major breakthrough for experimental and clinical investigators was the development of charcoal yeast extract agar supplemented with 1-cysteine and ferric pyrophosphate (92). Addition of ACES buffer (202) and alpha-ketoglutarate (67) has improved growth of legionellae even further. The active principles in yeast extract appear to be purine and pyrimidine derivatives, of which guanine is the most efficacious (204). Hoffman and associates have demonstrated that the critical role of activated charcoal probably derives from its ability to scavenge toxic oxygen radicals that are produced when yeast extract agar is exposed to light (130). Albumin (141) and starch (225) have been substituted for charcoal by some investigators. Keto acids, such as alpha-ketoglutarate, may perform a similar function by stimulating production of bacterial reduced-oxygen-scavenging enzymes (206).

From the early days in which growth factors were demonstrated empirically, we have now developed a sophisticated and rational understanding of the metabolism of legionellae. One might speculate whether one or more of the factors, such as guanine, is supplied efficiently to the bacteria in the intracellular environment.

Legionella was born in the molecular age, and the sophisticated resources of molecular biology have greatly expanded our knowledge of the biochemical composition of the genus. All of the legionellae have the ultrastructural appearance of gram-negative bacilli, including inner and outer membranes. *L. micdadei* has a distinctive dense layer in the periplasmic space (123). This dense layer appears to be expressed differentially according to conditions of culture; its relationship to the variable acid fastness of this species is unclear (118).

All of the species, except *Legionella oakridgensis* are motile. Flagella have been demonstrated on *L. pneumophila* both in vitro and in vivo (38, 218), and fimbriae are also present (218). The presence of an extracellular acid polysaccharide layer has been demonstrated by ruthenium red staining (123).

Gram-negative bacteria characteristically contain lipo-

polysaccharide (LPS) with endotoxic function. Johnson and colleagues reported an easily extractable high-molecular-weight compound that was composed primarily of lipid and had endotoxinlike activity (144). This F-1 antigen inhibited serospecific serological reactions, served as an opsonin, and was localized on the surface of the bacteria (80). The serospecificity of the antigen was confirmed after it was purified (45, 198). The LPS of serogroup 1 *L. pneumophila* is tightly bound to the major outer membrane protein (MOMP) (129). Most of the antibody demonstrated by indirect immunofluorescence in patients infected with serogroup 1 *L. pneumophila* appears to be directed against the LPS (107, 129). Cross-reactions with the lipid A of *Salmonella minnesota* were demonstrated. The LPS is distinctly different, however, from that of enteric bacteria in biochemical characteristics (50) and endotoxic function (276).

The MOMP of *L. pneumophila* has a molecular weight of 24,000 to 29,000, depending on the procedure used for isolation (34, 107, 129, 192). The 29-kilodalton (kDa) protein isolated by Gabay and Horwitz formed ion-permeable channels with a selectivity for cations when the protein contacted lipid membranes; this outer membrane protein functioned similarly to the porins of *Escherichia coli* (106). In various studies the MOMP has been found to be associated with LPS (129) and peptidoglycan (106). Butler and colleagues have suggested that the MOMP exists as a 95-kDa complex that dissociates into four subunits (34).

The role of the MOMP in pathogenesis and immunity is unclear. It is of interest that the MOMP in *L. pneumophila* appears to be extensively cross-linked by disulfide bonds (34, 107, 129), a property that it shares with *Chlamydia* species, which are also intracellular pathogens. The 24-kDa protein is immunogenic (89), but antibodies detected by indirect immunofluorescence of convalescent human sera appear to be directed primarily at the LPS component (107, 129). By iodine labeling of surface proteins (107) and radioimmunoprecipitation (89), the MOMP appears to be exposed on the surface of the bacterium. A 29-kDa protein that reacts with a commercial monoclonal antibody (117) and appears to be the MOMP (129, 192) was only partially exposed. Several investigators have shown that the 24- to 29-kDa protein from *L. pneumophila* reacts with most of the serogroups of this species and cross-reacts with proteins from other *Legionella* species (34, 117, 129, 192). Engleberg and colleagues were unable to detect cross-reactions between the 24-kDa protein and a variety of other gram-negative bacilli (89), but the 26-kDa outer membrane protein identified by Sampson and associates appeared to react nonspecifically with human sera (226).

A variety of cellular proteins are present in *L. pneumophila*. Many are present on the cell surface (107) and react with human sera (226). Proteins other than the MOMP are also candidates for species-specific or genus-specific reagents (89, 226). Understanding of the molecular basis for pathogenicity and development of better diagnostic reagents will both depend on continued dissection of bacterial genetics and biochemistry. Cloning of *Legionella* antigens into *E. coli*, which has been accomplished by several groups (65, 88, 154), has already been used to study membrane proteins.

Some but not all isolates of *L. pneumophila* contain plasmids (4). Serogroup 1 *L. pneumophila* has been dissected into a variety of subtypes by analysis of plasmid content (166), reactivity with polyclonal (253) and monoclonal (147) antibodies, and electrophoretic analysis of structural genes for bacterial enzymes (229).

Legionella species contain hemolysins (5) and proteases (66). The purified protease of *L. pneumophila* dissociates into two species with molecular weights of 38,000 and 40,000. The protease resembles neutral zinc-containing metalloproteases of other bacterial species. Other bacterial products, such as a toxin (164) in *L. pneumophila* and an acid phosphatase in *L. micdadei* (223), may also be important. Chen and colleagues have demonstrated that restriction endonucleases are produced (40).

The immune response to *L. pneumophila* is both humoral (101) and cellular (103, 132, 208). As discussed above, some of the antigens that stimulate a humoral immune response have been identified, but the nature of the antigens that stimulate cellular immunity has not been explored.

Pathogenesis

L. pneumophila, *L. micdadei*, and presumably other species have many of the characteristics of facultative intracellular pathogens. In cell culture the bacteria grow well within the cytoplasm of macrophages (138, 264), but do not grow in the usual cell culture media. PMNL are not effective bactericidal cells, but do not support bacterial growth (139).

Uptake of *L. pneumophila* by PMNL requires the presence of antibody and complement (139). In contrast, *L. micdadei* is phagocytosed in the absence of specific antibody if the alternate pathway of complement is intact. The third component of complement can be demonstrated by immunofluorescence on the surface of *L. micdadei* cells that have been exposed to serum (235), but not on similarly treated cells of *L. pneumophila* (139).

The macrophages of both animals and humans phagocytose legionellae in the absence of antibody, but are more efficient in its presence. Direct penetration by bacteria into the cytoplasm has been suggested (151), but uptake of *L. pneumophila* by guinea pig macrophages is inhibited by cytochalasin D, suggesting a conventional phagocytic process (81). In human macrophages a peculiar "coiling" phagocytosis of *L. pneumophila* has been described (135). This process has not been observed in guinea pig macrophages (81), but a similar phenomenon occurred when phagocytes from fish engulfed yeast cells (176). An oxidative burst occurs at the time of phagocytosis, as demonstrated by reduction of nitroblue tetrazolium at the site of phagocytosis (141). The bacteria enter a phagosome that has a distinctive association with mitochondria and ribosomes, both in natural human infection (113) and in experimentally infected macrophages (133). In the presence of virulent *L. pneumophila*, phagolysosomal fusion (134) and acidification of the phagosome (137) are inhibited.

L. pneumophila pneumonia may be produced experimentally by inoculation of the bacteria into experimental animals intratracheally (272) or by aerosol (9, 15, 52). Guinea pigs are the most susceptible species that has been examined to date. They can be infected with as few as 129 colony-forming units and the 50% lethal dose ranges between 10^3 and 10^4 colony-forming units after aerosol administration. The resultant disease resembles human *Legionella* pneumonia in many respects. Rats and hamsters are easily infected, but rarely die. In contrast, mice are extremely resistant to infection, unless they are immunosuppressed (267). The initial site of bacterial replication in the lung is the alveolar macrophage (52). It is impossible to say, however, that growth is restricted to the intracellular environment in vivo, as it is in cell culture systems. Recruitment of polymorphonuclear neutrophils is temporally associated with curtailment of bacterial growth, but clearance of the bacteria from the lung

probably requires the contribution of both the humoral and cellular immune systems.

The cellular basis for bacterial virulence is unknown. Avirulent variants of legionellae can be produced easily in the laboratory (174), but there has been no single potential virulence factor that is present in a virulent strain and absent from its avirulent counterpart. We are still looking for the smoking gun. There are, however, several bacterial products that may play a role. *L. pneumophila* is susceptible to both the myeloperoxidase and xanthine oxidase pathways in cell-free systems (164, 165), a susceptibility that may explain the limited bactericidal capacity of human PMNL (139) and the initial killing of a portion of the inoculum by monkey macrophages (141). Jepras and Fitzgeorge noted that virulent strains were resistant to killing by the xanthine oxidase system, whereas less virulent isolates were killed (142). Other investigators have found the two systems equally effective against avirulent and virulent isolates (165), so the issue remains unresolved.

L. pneumophila produces a toxin that inhibits the bactericidal activity of human PMNL against *E. coli* (105), but the toxin is produced by both virulent and avirulent isolates. An acid phosphatase from *L. micdadei* blocks production of superoxide anion in human PMNL; the nature of the strains used in the study was not described (223).

The protease is a candidate for tissue damage produced by legionellae. Early on, several investigators suggested the possibility of a toxin to account for the prominent extrapulmonary symptoms and the cytolytic inflammatory response (104, 273). The protease from *L. pneumophila* produces a necrotic reaction in the subcutaneous tissue of infant mice (14) and a necrotizing inflammatory response in the lung (8). It is not clear how the quantities of protease used in the experiments relate to those produced during a pulmonary infection.

When searching for virulence factors and comparing virulent and avirulent strains, it is important to ensure that sufficient numbers of strains from diverse localities are obtained so that a valid conclusion is made. It is also worth noting that the assessment of virulence is based on infectivity for animals rather than humans. Some potentially important factors, such as the presence of flagella, production of toxin and protease, and sensitivity to killing by serum, have turned out to be shared by virulent and avirulent isolates.

Recovery and Immunity

The mechanisms for resistance to pneumonic *Legionella* infection and for recovery from clinical disease are incompletely known. There is obviously a large host factor in the equation, because the attack rate is low in common-source epidemics and the fatality rate is not high in previously normal individuals. Well-defined clinical risk factors have been documented epidemiologically (85, 101, 122). Most prominent among these risk factors have been immunosuppression by a variety of means, chronic cardiopulmonary disease, and cigarette smoking. Neutropenia per se has not been documented as an important risk factor for *Legionella* infection, as it is for *Pseudomonas* and *Aspergillus* infections.

Most experimental data suggest that the cellular immune system is critical to recovery. Naive macrophages may kill some ingested legionellae (141), but subsequent growth is luxuriant. Macrophages from either guinea pigs or humans that are "activated" by treatment with lymphokines do not support bacterial growth, although they do not kill the ingested legionellae (103, 189). At least one of the active principles in the lymphokine preparations is gamma interferon, which can also activate macrophages to inhibit growth of *L. pneumophila* in vitro (18). It is interesting to note that the alveolar macrophages of mice, a very resistant animal species, are the exception to the rule that intracellular growth of legionellae occurs in this type of cell (278).

There is evidence from both in vitro and in vivo experimental systems that activated macrophages are nonspecifically armed against *Legionella* spp. If human monocytes from patients with leprosy are activated with supernatants from concanavalin A-stimulated lymphocytes, the monocytes inhibit the intracellular replication of *L. pneumophila* (136). Similarly, when a cellular immune response is produced in the lung by infection with mycobacteria, increased resistance to inhaled *L. pneumophila* results (111).

The role of antibodies in host defenses is less clear than that of cellular immunity. From in vitro studies, one might hypothesize that antibody could be detrimental by enhancing phagocytosis without inhibiting bacterial replication in susceptible macrophages. Experimentally, however, rats were protected against subsequent intraperitoneal infection by passive transfer of antibody (220). Similarly, antibody modulated a primary aerosol infection in guinea pigs (J. A. Elliott, W. C. Winn, Jr., and G. S. Davis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B-77, p. 37). When guinea pigs were given hyperimmune or normal serum before infection, bacterial multiplication was similar in the two groups over the first 24 h, but subsequently multiplication was inhibited in the animals that had received immune serum.

The full explanation for recovery from *Legionella* infection is not yet known. The most effective defenses identified to date result only in bacteriostasis or very limited killing. The effective defense mechanism may not yet have been identified, or a combination of factors may operate in concert in vivo.

It is unclear whether humans who have had Legionnaires disease are then immune to reinfection. Guinea pigs that have been immunized with killed *L. pneumophila* antigen are protected against subsequent intraperitoneal challenge, but not against aerosol infection (79). Surprisingly, Baskerville and co-workers were unable to protect guinea pigs that had been previously infected by aerosol against subsequent inhalation of a bacterial aerosol (10); in fact, the previously infected animals died somewhat sooner than their naive counterparts. In our laboratory, aerosol infection of guinea pigs with a sublethal inoculum of *L. pneumophila* has provided solid protection against infection 1 month later by either aerosol or the intratracheal route (W. Winn and G. Davis, unpublished observations). The immune animals have increased numbers of macrophages in the airspaces, and bacterial multiplication is depressed from the very outset of the infection.

LABORATORY DIAGNOSIS OF *LEGIONELLA* INFECTIONS

From the frustrating early days when *Legionella* infections could only be diagnosed retrospectively by serological techniques or by inoculation of guinea pigs, we have advanced to the point where it may be easier to identify with reasonable confidence infections caused by legionellae than those caused by more venerable pathogens, such as *Streptococcus pneumoniae*. Life has been complicated by the burgeoning list of new serogroups and species, but fortu-

nately a few serogroups and species predominate in human infections and a carrier state has not been demonstrated in humans. Development of genus-specific reagents will further increase diagnostic capabilities.

Infections can be diagnosed by demonstration of a serological response to the bacteria, by recognition of bacterial antigen or nucleic acid in clinical specimens, and by recovery of the pathogen in culture.

Culture

The mainstay of diagnosis at present should be culture (75, 275, 282). The sensitivity of culture is 50 to 80%, but the specificity is 100% and an isolate is then available for molecular analysis if epidemiological investigation is in order. BYCE-a agar is the mainstay of microbiological diagnosis. Selective versions have been formulated to assist in the recovery of legionellae from contaminated oral secretions (203); if species other than *L. pneumophila* are sought, a medium with vancomycin rather than a cephalosporin should be used. Acid treatment of sputum specimens may also increase the yield of legionellae, as it does for environmental samples (33).

Dye-containing media have been developed to assist in the recognition and preliminary identification to species of legionellae (259), but it is not proven that they are essential. The appearance of legionellae on the surface of charcoal agar is characteristic; the colonies are multifaceted and have been described as resembling cut glass (Fig. 2). Examination of the plates with a stereomicroscope speeds detection of isolates.

The media for growth of legionellae are commercially available as poured plates or in powdered form. The commercial media have functioned well, but there is considerable variation from company to company. We have encountered commercial media that did not support the growth of fresh clinical isolates, although stock laboratory strains might grow if inoculated heavily.

At least 2 days are usually required for recognition of colonies, but most cultures are positive within 5 days of incubation at 35°C. *L. pneumophila* grows well in air, but other species may require low concentrations of CO_2, not to exceed 5%.

L. pneumophila was initially isolated from blood by using a biphasic medium (74). This species has been recovered by using a commercial radiometric blood culture system (43, 216). In one case, a low positive growth indicator suggested the possibility of legionellae; in the other, daily blind subculture was required. A commercial lysis-centrifugation system was successful in recovering *L. pneumophila* from seeded blood cultures (59), but clinical isolates have not been reported.

Once a possible *Legionella* sp. has been isolated on a charcoal agar plate, subcultures should be made in parallel to another BCYE-a plate and to a BCYE-a plate without cysteine or a blood agar plate. The cysteine-deficient plate has some advantages, because *L. pneumophila* may grow on some types of blood-containing agar (P. J. Dennis, J. A. Taylor, and G. I. Barrow, letter, Lancet **ii**:636, 1981). In addition, thermophilic bacilli that mimicked the colonial morphology of *Legionella* spp. and did not grow on sheep

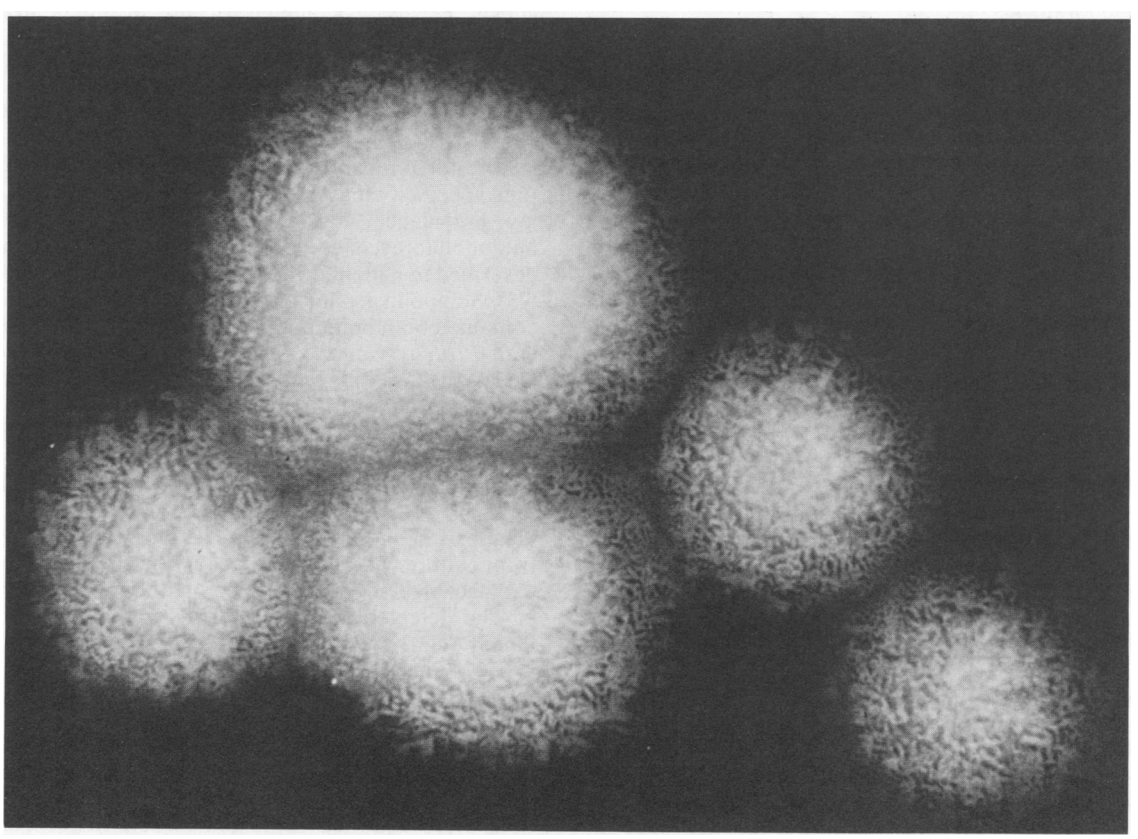

FIG. 2. Colonies of *L. pneumophila* on BCYE agar, as viewed with a dissecting microscope. The patterned surface of the colonies, which has been described as resembling cut glass, suggests the proper identification of the isolate. Magnification, ×16.

blood agar have been encountered (247). Fortunately, the thermophilic isolates did not react with antisera to *Legionella* spp., but the possibility of cross-reactions exists. On the other hand, *Legionella* spp. do not grow well on sheep blood agar, which is an inexpensive and easily accessible medium. Bacterial species that are cysteine dependent, such as *Francisella tularensis*, may be isolated on BCYE-a agar, after which subculture onto either blood agar or cysteine-deficient medium might suggest the possibility of a *Legionella* sp. (265). An isolate that does not react with available antisera might represent a new serogroup or species of *Legionella*; such cultures should be referred to a reference laboratory.

The morphology of the isolate should be examined by Gram stain. This family of bacteria is relatively inert biochemically. As Brenner has noted, the phenotypic characteristics of most species are based on a very small number of isolates (27). Furthermore, not all isolates give the expected results. In most clinical laboratories serological documentation of reactivity with antiserum to *Legionella* spp. provides adequate confirmation of the diagnosis (250). Cross-reactions with other bacteria have been reported, but are not common. Benson and colleagues noted that some strains of *Bordetella pertussis*, which may be isolated on BCYE agar, reacted with antisera to serogroup 10 *Legionella pneumophila*, *Legionella maceachernii*, *Legionella gormanii*, and serogroup 1 *Legionella feelei* (12). Neither serology nor analysis of fatty acid composition can absolutely discriminate among species, as was demonstrated when multilocus enzyme analysis revealed that a group of isolates, initially identified as serogroup 1 *L. pneumophila*, probably represented a new species (76). Genetic analysis is the final arbiter, but these techniques are at present restricted to reference laboratories.

Detection of Bacterial Antigen and Nucleic Acid

The first method for direct detection of bacterial specimens in clinical specimens was immunofluorescence. The sensitivity of direct immunofluorescence is low, approximately 25 to 50%, but a diagnosis can be achieved rapidly. The specificity is very high and the test may be useful in an epidemic setting. When the prevalence of disease is low, however, the predictive value of positive results may be unacceptably low. Cross-reactions with other bacteria by direct immunofluorescence have been demonstrated (195). These cross-reactions have not been frequent in clinical specimens, but have been reported (D. A. Johnson, K. F. Wagner, J. Blanks, and J. Slater, letter, J. Am. Med. Assoc. **253**:40–41, 1985). A more serious problem is contamination of reagents with environmental legionellae. Pseudoepidemics of *Legionella* infection have resulted from falsely positive direct immunofluorescence tests, so that all reagents should be filter sterilized (217). Commercial reagents for the test are available. A monoclonal species-specific antibody is comparable in sensitivity to polyclonal conjugates; the monoclonal antibody does not cross-react with pseudomonads that react with polyclonal conjugates (243). In a retrospective study of clinical specimens, the performance of the monoclonal and polyclonal reagents was similar, but less background staining was observed with the monoclonal reagent (69).

Recently, a commercial nucleic acid hybridization test for *Legionella* ribosomal ribonucleic acid that is genus specific has been marketed. The probe was able to identify all of 156 strains of *Legionella* spp. correctly and excluded 106 other gram-negative bacilli (68). When the DNA probe was applied to 112 frozen clinical specimens, from which *Legionella* spp. had been isolated, the sensitivity was less, ranging from 57 to 74% depending on the method of analysis. Thus, this potentially important molecular tool performs as well or better than direct immunofluorescence and could be used as a tool for rapid diagnosis. It cannot replace culture or be used to screen specimens for subsequent culture, however. One disadvantage of the present kit is that a radiolabeled probe with a short half-life must be used. An experimental probe prepared from sequences within the gene that encodes the MOMP has been used to detect *L. pneumophila* in the lungs of infected mice (87).

Detection of *Legionella* antigen in urine has also been accomplished by radioimmunoassay, enzyme immunoassay, and latex agglutination (227). The sensitivity of the radioimmunoassay and enzyme immunoassay was approximately 80% under optimal conditions, and the specificity was very high. The latex test was less sensitive, but considerably more rapid and convenient. Protracted antigenuria occurred in some patients. The lengthy excretion of antigen is a potential advantage in that a diagnosis can be made late in the course of infection, but a potential disadvantage in that a positive test may reflect a prior, rather than a current infection. The reagents for these tests are not yet commercially available.

Serological Diagnosis

A variety of serological tests have been developed for detection of antibodies to *Legionella* spp. Of these, the indirect immunofluorescence test has been the most completely evaluated. Several methods of preparing the antigen appear to produce similar results if the breakpoints for determination of positivity are adjusted (268). It is important to use reagents that detect both immunoglobulins M and G (188, 281).

The sensitivity of the fluorescence test is approximately 75% (75, 269), and the specificity approaches 100% (269). As is true for antigen detection methods, however, less than total specificity may produce problems when the disease has low prevalence. Falsely positive reactions in the indirect immunofluorescence test for *L. pneumophila* have been described in patients who had *Bacteroides fragilis* infections (73). Sompolinsky and associates have encountered sera that contained both immunoglobulin M and G antibodies reactive with *Proteus vulgaris*, *Rickettsia typhi*, and *L. bozemanii* (232). Agglutinating antibodies that cross-react with *Pseudomonas aeruginosa* in patients with cystic fibrosis have also been reported (49). As the number of antigens tested in serological tests increases, the criteria for positivity should be increasingly strict (270). A fourfold increase in titer to a level of at least 1:128 is the standard. Even then, the diagnosis of sporadic cases by serology is not as firm as that from culture. Serological diagnosis has the additional disadvantage of being retrospective, although a seroconversion can be detected in many patients within the first week of illness (282).

EPIDEMIOLOGY AND ECOLOGY

The epidemic nature of *Legionella* infections was established from the outset (101), but it was soon apparent that sporadic infections also occurred (173) and that many infections were acquired in hospitals (32). In the past decade, outbreaks of Legionnaires disease have occurred on three of the five continents (7), and sporadic cases number in the

thousands (85). Although not all species have been isolated from humans, each should be considered a potential pathogen. The vast majority (85%) of human pneumonic infections have, nevertheless, been caused by *L. pneumophila*, particularly serogroup 1 (214); *L. micdadei* contributes an additional 6% of the cases. Pontiac fever has been caused by *L. pneumophila* serogroups 1 (114) and serogroup 6 (167, 233) and by *L. feelei* (128).

The frequency of *Legionella* infections is difficult to assess. Retrospective seroepidemiologic surveys are flawed, because they do not utilize all diagnostic modalities and because the criteria for testing may introduce bias. Among 500 patients with community-acquired pneumonia in Seattle, 5 (1%) developed at least a fourfold rise in antibody to *L. pneumophila* serogroup 1; 4 of the patients also developed antibodies to influenza virus A (98). In Iowa, 4.1% of 586 patients with pneumonia developed antibodies to *L. pneumophila* (215).

Prospective comprehensive studies have also been flawed, no matter how carefully designed, because of the almost insurmountable difficulties in establishing a definitive cause for most pneumonias. In some studies the inclusion of multiple diagnostic modalities (serology, direct immunofluorescence, and culture) for *Legionella* spp. and limited testing for other bacteria may have inflated the frequency of *Legionella* infections. On the other hand, inclusion of immunologic testing of sputum for pathogens that are also normal flora, such as *Streptococcus pneumoniae*, can also be criticized. In several series *L. pneumophila* has been a very common, if not the most common, cause of both community-acquired and nosocomially acquired pneumonia (169, 266, 279).

It is clear that a careful search for *Legionella* infections must be made before clinicians and laboratory workers can conclude that the bacterium is not a problem in the hospital or community (184). Documented absence of a clinical problem does not assure its continued absence. Between the two large epidemics in Burlington, Vt., there were 2 years in which only a small number of sporadic cases occurred, probably from a source different from the epidemic site (148). There have also been several retrospective surveys of fatal pneumonias in patients at high risk of developing Legionnaires disease in which a very low incidence was documented (115, 240).

Colonization of humans by legionellae has not been demonstrated by culture (31). Person-to-person transmission has not been documented (280), and hospital personnel do not appear to be at risk of contracting infection from patients during an epidemic (168), although they may be at risk for contracting infection from an environmental source in or near the hospital.

The only environmental sources of *Legionella* infection that have been identified to date have been related to water. The bacteria can be recovered from surface waters that have a variety of physical, chemical, and biological characteristics and are widely separated geographically (96, 97). Legionellae have been isolated from mud, but not from dry soil. The first clue that water in air-handling devices might be a source of infection came from the Pontiac fever outbreak (114). Legionnaires disease was associated with the drift from a cooling tower in Memphis, Tenn. (57). Contaminating bacteria in a cooling tower could come from the input water or from the outside environment. The first clue that potable water might be an important source came from a renal transplant unit in Oxford, England, where *L. pneumophila* was isolated from shower stalls that were associated epidemiologically with infection in patients who had received transplants (255).

The evidence for cooling towers and evaporative condensers (185) and potable water (99) has been recently reviewed. The role of air-handling equipment in Pontiac is indisputable, as is the source of infection in two maintenance workers who entered a cooling tower in Burlington, Vt. (112). Epidemiologic evidence from several epidemics provides a strong association of cooling towers with epidemic disease (57, 157). Although some cases in these epidemics do not fit neatly into the cooling tower pigeonhole, epidemiological associations are rarely absolute.

The epidemiologic association of potable water with disease is also strong (16). The experience of the Wadsworth Veterans Administration Hospital in Los Angeles, Calif., is most impressive. Although cooling towers at the institution contained legionellae, decontamination of the towers had no effect on a continuing epidemic. Subsequently, potable water was shown to be the probable source of the bacteria, and decontamination of the drinking water produced a dramatic reduction in the number of cases (230). Other hospitals have had a similar experience (127).

As is true with air-handling equipment, the most direct evidence for potable water as an important source of infection has come from analysis of small clusters of cases. Arnow and colleagues were able to document the occurrence of Legionnaires disease in patients who were exposed to humidifiers and nebulizers that had been filled with *Legionella*-containing tap water (3).

In an epidemic, multiple sources for the bacterial inoculum must be considered. As has already been mentioned, investigators should not assume that air-handling equipment represents the only source.

Molecular tools for subtyping of isolates can be of help in sorting out the role of multiple environmental sources (108, 147, 193). Edelstein and colleagues found that multilocus enzyme analysis divided a collection of isolates into the greatest number of subtypes of *L. pneumophila*, but plasmid analysis and monoclonal antibody testing were also useful (76). Molecular tools may even suggest more than one source for clinical cases (148); monoclonal antibody typing of isolates from Burlington, Vt., demonstrated a commonality between epidemic isolates and strains from a cooling tower, while sporadic strains matched isolates from drinking water in the hospital.

Even sophisticated typing studies can only differentiate among different strains or subtypes. It is impossible to prove absolutely that two isolates with identical characteristics are, in fact, the same. In addition, as typing schemes become more complex and split strains into increasingly more numerous subgroups, it will become more difficult to decide when two strains with slightly different reactivity are, in fact, distinct.

Epidemiologic analysis of epidemic and sporadic cases has identified a variety of risk factors for the development of Legionnaires disease or for fatal infection. Notable among these have been cigarette smoking (101), advanced age (85), chronic lung disease (85), and immunosuppression (83, 122). Renal transplant patients have been at particularly high risk in several institutions (22, 63, 170). Although Legionnaires disease has been associated epidemiologically with increasing age, pediatric infections have been described (51, 158). It is likely that a combination of risk factors produces the highest probability of infection: decreased host defenses and exposure to an environmental source that is disseminating virulent bacteria (3).

Legionella species are ubiquitous in the environment. They have been isolated frequently in the absence of human disease (54, 97). Ehret and colleagues have reported a monoclonal antibody that distinguished between strains isolated from humans and those from potable water sampled at random (78). It is not known whether the differences reflect variation in virulence or whether isolates might vary in biochemical composition depending on the growth medium (tissue versus water). Testing of strains from a wide geographic distribution and environmental strains that have been associated with human disease will be interesting.

Although most work has been done on *L. pneumophila*, several other species have produced infections that have been associated with potable water (16, 146, 200).

Even after the recognition of aquatic environments as important factors in human disease, the mechanisms by which survival and dissemination of legionellae occurred were a mystery. Our knowledge of interrelationships between the bacteria and the environment has increased greatly over the past 10 years, but much needs to be learned about the relative importance of the various factors that have been identified. This progress has been made possible by the development of selective media for environmental specimens (203, 261) and of an acid wash procedure for reducing other environmental bacteria (25). The selective media are now more effective in isolation of legionellae from water than is intraperitoneal inoculation of guinea pigs (77).

L. pneumophila in potable water has been isolated most frequently from the sediment in hot-water tanks and from peripheral plumbing fixtures. It has been difficult to recover legionellae from municipal water supplies, even at the point of entry into buildings that are heavily colonized (230). Although organisms have been recovered from cold water (238), isolation from hot-water systems has been more common.

There are several bacterial characteristics that may enhance its ability to survive and even to thrive in water systems.

Interaction with Other Microbes

The first suggestion of symbiosis between *Legionella* species and other organisms came from the isolation of *L. pneumophila* from an algal mat in a thermally polluted lake (254). When algae are removed from a culture that contains *Legionella* spp., the numbers of viable *Legionella* spp. in the mixture decrease (23).

L. pneumophila does not grow in sterile tap water. Naturally occurring legionellae do multiply, however, when they are associated with other environmental bacteria (262). Satellite growth of *L. pneumophila* could be demonstrated around the environmental isolates in cysteine-deficient medium.

Rowbotham was the first to document the phagocytosis of legionellae by environmental amoebae (221). Subsequently, multiplication of the bacteria in several species of free-living amoebae has been demonstrated (6, 131, 256). In one instance, *L. pneumophila* and two protozoan species, *Tetrahymena* sp. and *Cyclidium* sp., were isolated from cooling-tower water; in the laboratory, both protozoan species supported the intracellular growth of the *Legionella* isolate (6).

Effect of Chlorine on Environmental Bacteria

Legionella pneumophila is more resistant to the bactericidal effects of chlorine than are enteric bacteria, which provide the standard by which the quality of potable water is assessed in the United States. At 21°C, pH 7.6, and 0.1 mg of free chlorine residual per liter, a 99% kill of *L. pneumophila* required exposure for 40 min, whereas *E. coli* was killed in <1 min (161). The demonstration that environmental legionellae lost their chlorine resistance after subculture on agar media is an important caveat for future investigators of this problem (160). Furthermore, "super" chlorine-resistant strains have been isolated from plumbing fixtures. One of the reasons for the frequent association of legionellae with hot water may be the difficulty of maintaining adequate levels of chlorine at elevated temperatures.

Effect of Temperature and Characteristics of Water

L. pneumophila can multiply in tap water at temperatures as high as 42°C, whereas enteric bacilli are inhibited at the elevated temperatures. The bacteria survive even higher temperatures. *L. pneumophila* has been isolated from hot-water tanks that were maintained at temperatures between 30 and 54°C, but not from tanks held at 71 to 77°C (263). At 50°C there was little loss in viability of *L. pneumophila*; in contrast, a *Pseudomonas* sp., a *Micrococcus* sp., and a coliform survived less well (53). *Legionella* species other than *L. pneumophila* varied in their resistance to elevated temperatures. Some, such as *L. bozemanii*, survived better than *L. pneumophila*, whereas *L. micdadei* fared less well.

The temperature stability of the legionellae may explain the finding that legionellae survive less well in oil and gas water heaters, in which the heating element is at the bottom of the tank adjacent to the sediment, than in electric heaters, where the heating element is in the middle and the sediment at the bottom is not exposed to the highest possible temperatures (145). In one study, legionellae were more frequently isolated from warm whirlpools (35 to 40°C) than from swimming pools (8 to 30°C) (119).

The importance of individual components of plumbing systems was first suggested by Colbourne and colleagues, who isolated legionellae from rubber fittings during investigation of a nosocomial outbreak in England (47). Niedeveld and colleagues found that growth of *L. pneumophila* was stimulated by rubber compounds that did not contain thiuram (191). In a model hot-water system, legionellae were found most frequently on rubber fittings, but not on copper fittings (228). A variety of metals are toxic to legionellae in drinking water, but some, such as iron and zinc, can stimulate growth when present in low concentrations (234).

It is entirely possible that a combination of factors are operative in plumbing systems. Stout and co-workers have demonstrated that the concentration of sediment was directly related to survival of legionellae, that the presence of environmental bacteria also improved survival, and that the two factors were additive (237). They have suggested that the failure of sediment and bacteria to stimulate the growth of *L. micdadei* may explain the relatively infrequent isolation of this species from potable water (17).

Route of Infection

Several potential routes exist by which the bacteria could be transferred to humans from the environment. Ingestion of bacteria followed by dissemination from the gastrointestinal tract has been suggested, but there is very little pathological evidence to support that possibility in humans. Pneumonia and lethal infection has been produced by feeding contaminated water to guinea pigs, but very large doses were

required and the bacteria were introduced directly into the stomach with a feeding tube (209). The disease that is produced after intraperitoneal inoculation of bacteria into guinea pigs resembles human infection less well than does infection by the respiratory route (151).

Aspiration of oropharyngeal contents has also been suggested as a possibility, because the frequency of nosocomial pneumonia has been low in patients who have had a laryngectomy and therefore no communication between the lower and upper respiratory tracts (143). It is difficult to exclude this possibility. Some members of the oral flora have been shown to stimulate growth of legionellae in the absence of cysteine (236), but other oral flora inhibit *Legionella* growth in vitro (95). In the absence of documented oropharyngeal colonization by legionellae, the bacteria would presumably come from aspiration while drinking.

Inhalation of a bacterial aerosol is the only well-documented method of infection, but the source in most sporadic cases that are associated with contaminated potable water remains unclear (Table 3). *L. pneumophila* has been isolated from aerosols created by water faucets (24), by humidifiers, and by the squeezing of manual ventilation bags (277). Some investigators have been able to demonstrate legionellae in aerosols produced by showers (24, 55), but others have been unsuccessful (277). In one study, *L. pneumophila* was isolated during two successive 15-min periods (24), whereas Dennis and colleagues were able to recover legionellae only during the first sampling period (55). Analysis of aerosols with cascade impingers has shown that the bacteria are contained in droplets that are of respirable size (55). In all of these studies very small numbers of bacteria were isolated, and it is not clear whether the inocula would be infectious for humans. Humidifiers and nebulizers, however, are proven sources for serious nosocomial infection (3).

Investigation and Control of Legionnaires Disease

Because of the ubiquity of legionellae in the environment, the lack of association of most isolates with human disease, and the adverse effects on humans or the environment of procedures designed to eliminate the bacteria (99), it seems most prudent to concentrate scarce resources on reliable documentation of human infection. Once human disease has been found, all possible environmental sources should be examined, using the assistance of molecular biology for the typing of isolates.

In many ways we are in a far better position to control *Legionella* infections than those caused by other gram-negative bacilli, which are part of the indigenous human microflora. Vivid testimony to that fact is the uniform, although sometimes temporary, success that many institutions have experienced in protecting their patients from Legionnaires disease. It is certain that those same institutions have not successfully controlled pneumonia caused by pseudomonads or enteric bacilli.

TABLE 3. Sources of human infection

Source	Legionellae demonstrated		Transmission suggested
	By culture	In aerosols	
Cooling towers	Yes	Yes	Yes
Evaporative condenser	Yes	Yes	Yes
Shower heads	Yes	Yes	No
Faucets	Yes	Yes	No
Nebulizers	Yes	Yes	Yes
Humidifiers	Yes	Yes	Yes
Whirlpools	Yes	No	Yes
Swimming pools	Yes	No	No
Aspiration of upper-airway flora	No	No	

Two treatment modalities, temporary elevation of water temperature (16) and chlorination (230), have been used most frequently for treatment of potable water systems. In some instances (94, 127), both methods have been used. The sediment in hot-water tanks should be drained. As we learn more about supportive and nonsupportive components of plumbing systems, it may be logical to replace those elements that encourage the growth of legionellae. Once treatment is instituted, microbiologic surveillance of the water must be continued, because resurgence of the bacteria is common and clinical disease may once more result (26, 120, 157). It has been suggested that dead-end loops in the plumbing system may represent sites in which legionellae can survive and later repopulate the system (44, 120).

Decontamination of cooling towers and evaporative condensers is particularly difficult, because of the complexity and openness of the environment. As with antimicrobial agents, in vitro testing of biocides against legionellae has not correlated well with culture results in real cooling towers (84). The search for effective agents continues (82).

FUTURE DIRECTIONS

It is unlikely that future progress will occur at the same rate that we have witnessed in the past 10 years. There remain several areas, however, in which breakthroughs are badly needed. More knowledge of the molecular biology of the legionellae will be an important tool for understanding more fully the nature of bacterial virulence and host defenses. Of particular practical import would be the definition of a bacterial characteristic that predicted infectivity for humans. If such a factor were found, a rational basis for sampling the environment of high-risk areas, such as hospitals, could be developed. Better diagnostic reagents and approaches may also come from continued investigation at the molecular level.

The anxious days when *Legionella* was a great unknown are well past, but the bacteria will never be eradicated from the environment and patients with decreased host defenses will always be with us. As long as this combination obtains, the risk of Legionnaires disease will remain.

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